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[54] **HYBRID GENETIC COMPLEMENT AND CORN PLANT DK671**

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[51] **Int. Cl.**[6] **A01H 5/00;** H01H 4/00; C12N 5/04

[52] **U.S. Cl.** **800/200;** 800/250; 800/DIG. 56; 435/240.4; 435/240.5

[58] **Field of Search** ..... 800/200, 250, 235, DIG. 56; 47/58, DIG. 4; 435/240.4, 240.49, 240.5, 240.45, 172.1, 172.2, 172.3

[56] **References Cited**

PUBLICATIONS

Phillips et al. (1988) Cell Tissue Cellfield and In Vitro Manipulation In Corn & Corn Improvement ASA Publication No. 18 3rd edition pp. 345–387.

Meghiji et al. (1984) Crop Science vol 24, pp. 545–549.

Sass (1977) *Corn & Corn Improvement* ASA monograph #18, 2nd edition, Editors Sprague et al. pp. 89–110.

Poehlman (1987) *Breeding Field Crops* AVI Publishing Co. pp. 237–246.

Armstrong and Green, (1985), "Establishment and Maintenance of Friable, Embryogenic Maize Callus and the Involvement of L–Proline," Planta, 164:207–214.

Duvick, D. N. (1984), "Genetic Contribution to Yield Gains of U.S. Hybrid Maize–1930–1980," Genetic Contribution to Yield Gains of Five Major Crops, pp. 15–48.

Gordon–Kamm, W. et al., (1990) "Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants," The Plant Cell, V. 2, 603–618.

Green, C. E. and Rhodes, C. A. (1982) "Plant Regeneration in Tissue Cultures of Maize".

Poehlman, J. M. (1987), Breeding Field Crops, Third Edition, pp. 469–481.

Rhodes, C. A., Pierce, D. A., Mettler, F. J., et al. (1988). Science 240:204–207.

Sprague, G. F., Eberhart, S. A. (1977) Corn and Corn Improvement Chapter 6–"Corn Breeding" pp. 305–362 24.

Troyer, A. F. (1990). "A Retrospective View of Corn Genetic Resources," Journal of Heredity, 81:17–24.

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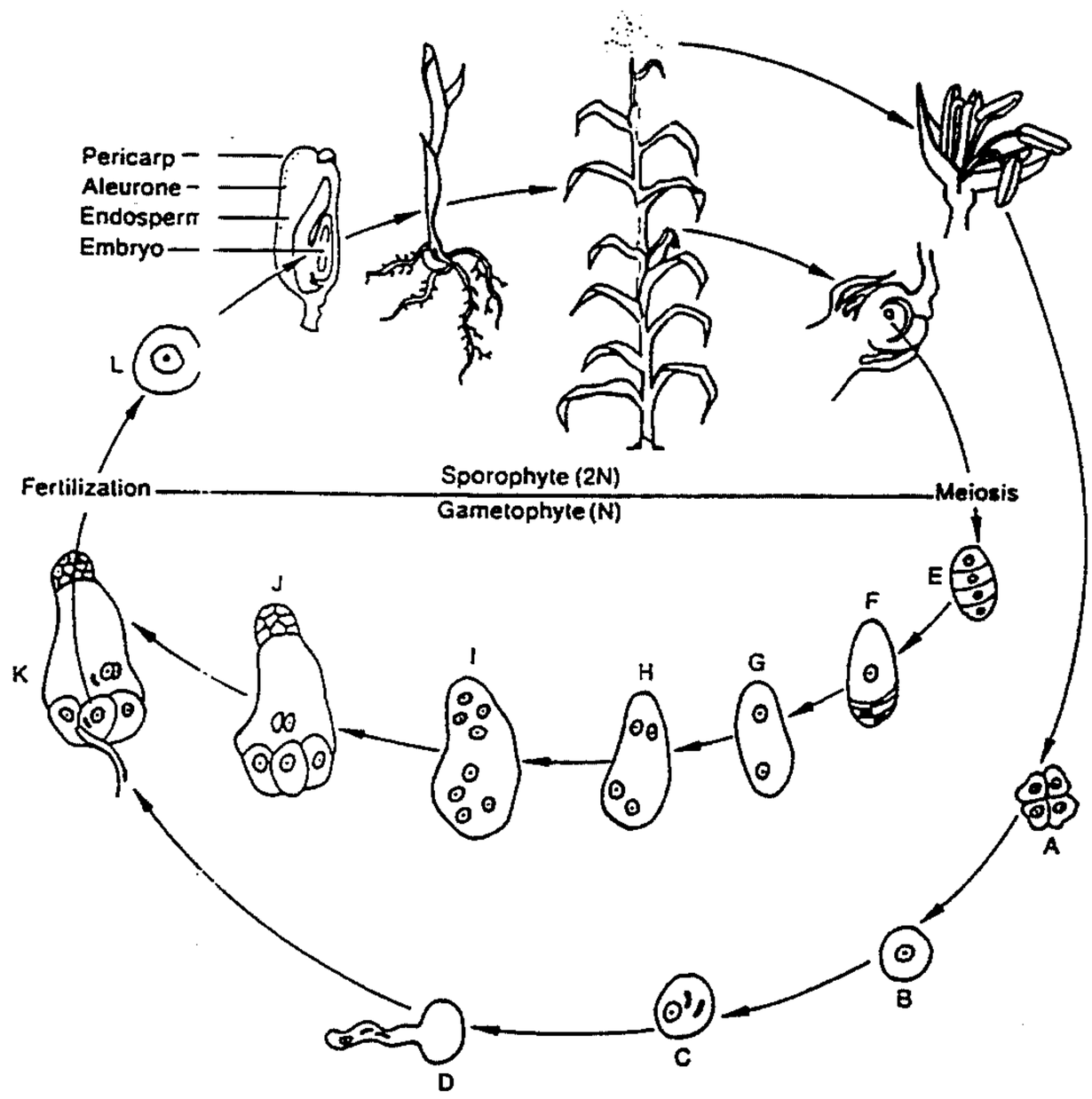
[57] **ABSTRACT**

This invention relates generally to the production of maize, commonly known in the United States as corn, and more specifically to hybrid corn plants with certain advantageous phenotypes resulting from interactions of the haploid genetic contributions of inbred parental lines. This invention relates to the hybrid genetic complement, the expression of which produces these phenotypes and to the complement as housed in seeds and tissues, in particular, those capable of producing or regenerating the hybrid plants either in vivo or in vitro. An aspect of this invention, hybrid DK671, is characterized by many advantageous phenotypic traits including superior yield, seedling vigor, stay green, stalks and roots. It has characteristic restriction fragment length polymorphism (RFLP) and isozyme profiles.

4 Claims, 2 Drawing Sheets



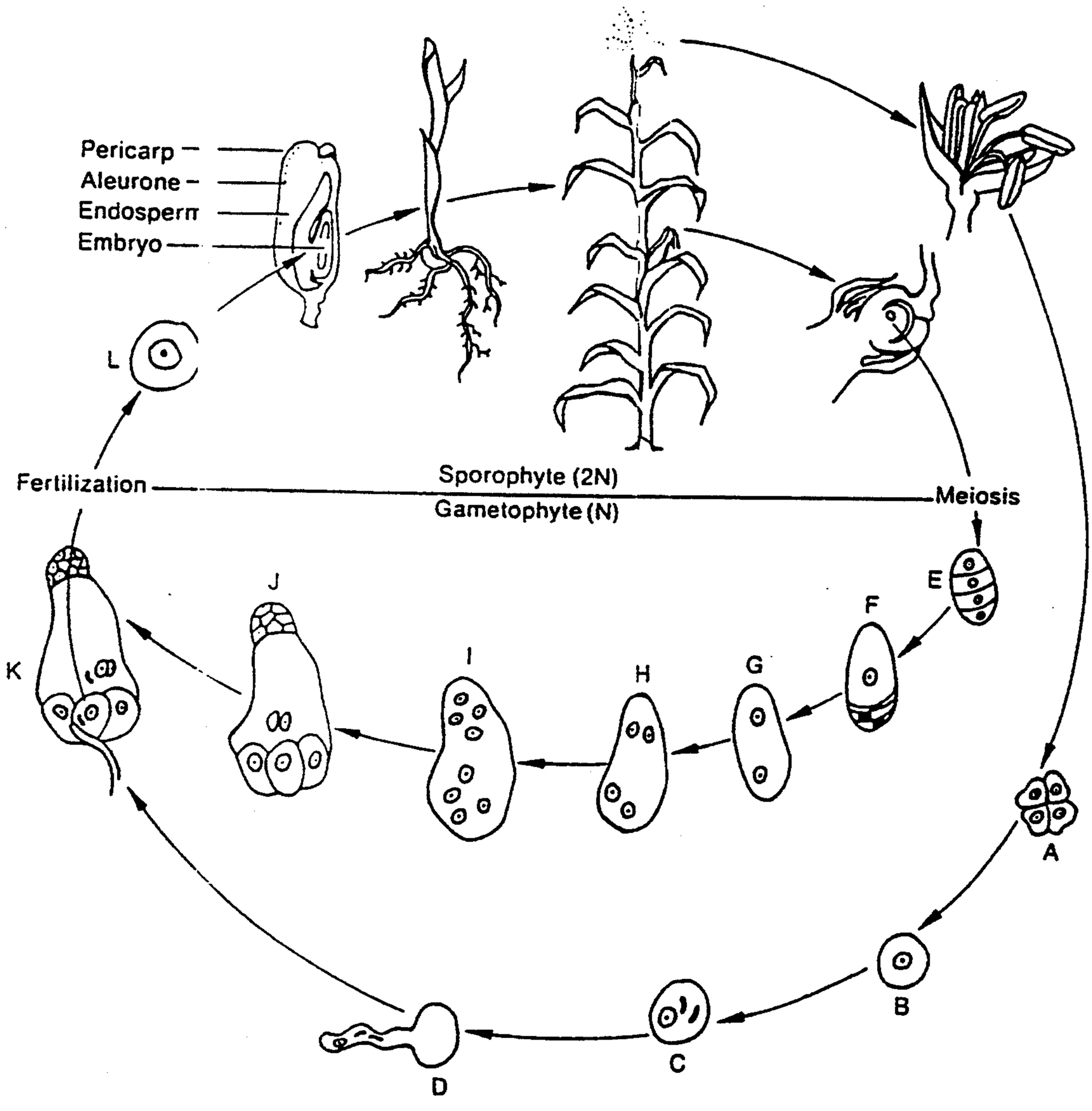

Pericarp
Aleurone
Endosperm
Embryo
L
Fertilization
Sporophyte (2N)
Gametophyte (N)
Meiosis
A
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FIG. 1

RELATIVE MATURITY MAP

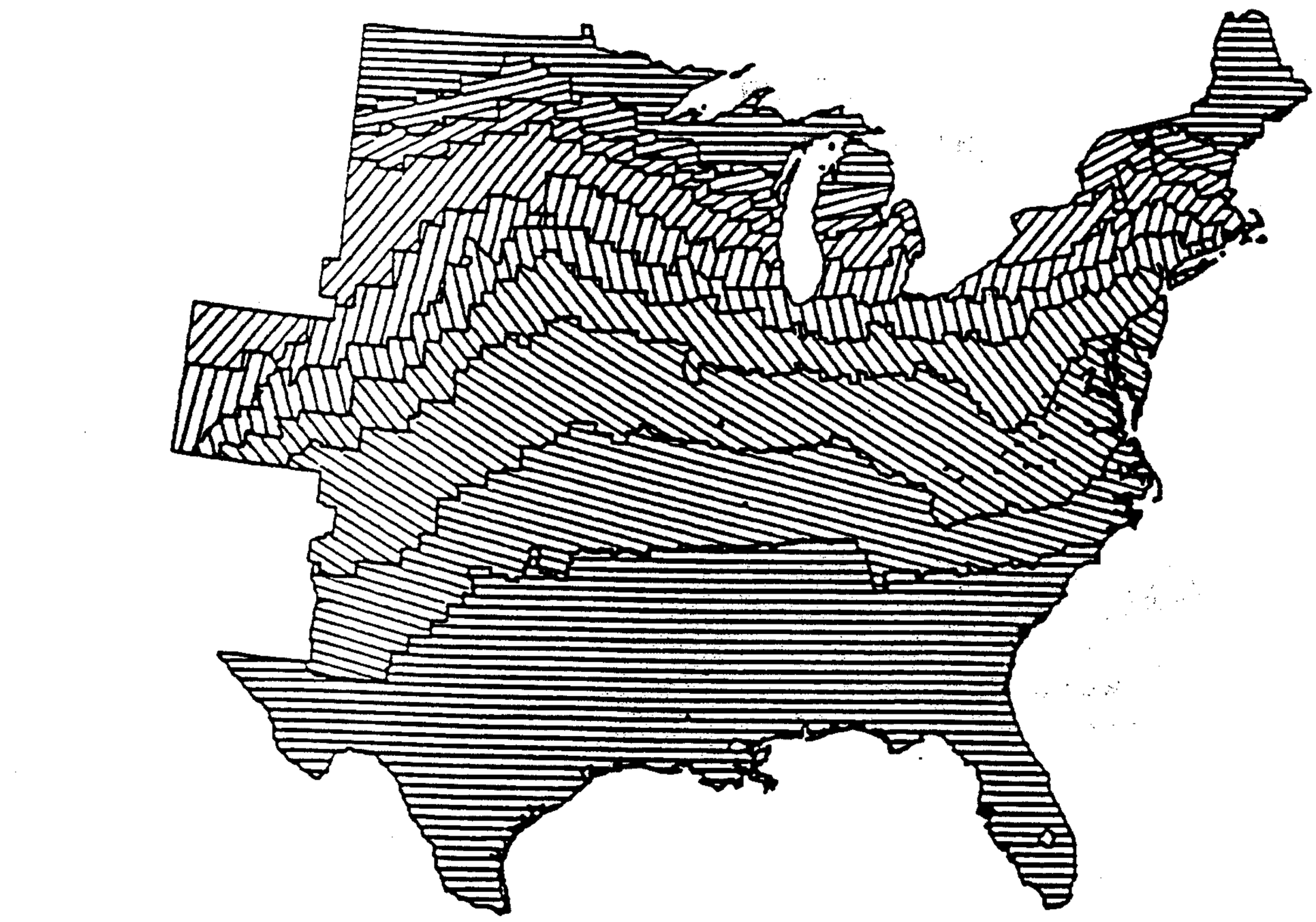

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FIG. 2

HYBRID GENETIC COMPLEMENT AND CORN PLANT DK671

FIELD OF THE INVENTION

This invention relates generally to the production of maize, and more specifically to hybrid corn plants with certain advantageous phenotypes resulting from interactions of the haploid genetic contributions of inbred parental lines. Seeds and tissues, in particular, those capable of producing or regenerating the hybrid plants either in vivo or in vitro are disclosed. An aspect of this invention, hybrid DK671, is characterized by many advantageous phenotypic traits including superior yield, seedling vigor, stay green, stalks and roots. It has characteristic restriction fragment length polymorphism (RFLP) and isozyme profiles.

DESCRIPTION OF THE RELATED ART

Crop improvement has been a major focus of human agriculturists since the hunting gathering societies moved into the agricultural phase of human existence. Early crude attempts to improve crops focused on the choice of parental plants to become the progenitors of the next generation, a choice made on the readily detectable characteristics of the parents. The objective was to produce offspring having the advantageous traits of the parents. However, from what we now know of genetics and genetic theory, such efforts were usually doomed to failure—in some instances either because the parental phenotypes could not be reconstructed in their offspring due to disruption of the genetic complements of the parents by segregation of a diploid complement into haploid gametes, and shuffling of the genetic material by recombination. Even worse, certain combinations of parental genomes yielded deleterious effects due to interactions of genes at the same or different loci. As a consequence, success at crop improvement was painstakingly slow, sporadic and rarely reproducible.

Modern sophisticated crop breeding of the 1900's has benefitted from knowledge gained by Gregor Mendel and others in the late 1800's indicating that both single gene (mendelian) and polygenic control must be considered when planning breeding programs to improve crop characteristics. In fact, all corn as we know it today, *Zea mays,* is a result of human manipulation. It was never a natural plant. Despite much knowledge that has developed subsequently, each breeding program represents at least in part a new attempt to mold the plant germplasm into new and more productive, more desirable phenotypes. This molding process benefits from the development over many years of inbred lines. These lines are not found in the wild, that is, in natural settings, and by themselves are generally not commercially productive. However, they are repositories for genes that are preserved in relatively stable conditions due to the true-breeding capabilities of these genetically uniform lines. Such genes are then available to be repeatedly tested for their effects in various breeding combinations and to be incorporated into commercially desirable crops.

Inbred lines are those that are essentially homozygous due to repeated breeding between ancestrally related lines (inbreeding) which concentrates a subset of ancestral genes in offspring. Homozygosity refers to the condition of the genetic complement in which the paired diploid positions at each locus are occupied by identical alleles. Alleles are conditions of a gene which

differ in their nucleotide sequences. Homozygosity in an inbred line is achieved by repeated inbreeding. In general, by the sixth or seventh generation, the inbred line is considered genetically pure, or "true-breeding" although spontaneous changes in the genetic material (mutations) and other events may preclude absolute homozygosity. Environmental variations may produce phenotypic variability.

Unfortunately, reduction in yield performance and the appearance of other plant characteristics which are undesirable accompanies inbreeding. In addition, progressive selfing of inbred lines reduces plant vigor. Many of these deleterious effects are caused by homozygosity for deleterious recessive genes whose effects are unmasked by loss of desirable dominant alleles. Consequently, inbred corn lines per se are not grown to be used as commercial crops. However, they are extremely important as vehicles to preserve genes and to produce first generation (F_1) hybrids by the process of hybridization by cross-breeding. Hybrid plants are likely to be heterozygous at many loci, as opposed to being homozygous, in contrast to the inbred parental lines. Heterozygosity refers to the fact that at a locus, there are different conditions of a gene (different alleles). One desirable result of crossing two inbred lines is that hybrid vigor or heterosis may arise wherein the hybrid plants produced have markedly improved higher yields, better stalks, better roots, better uniformity and better insect and disease resistance. For corn used as animal feed, one of the goals is decreasing the amount of feed needed for animal weight gain.

Furthermore, as result of self-pollination of these F_1 hybrid plants, a process possible in plants such as corn which have both male and female sex organs on the same plant (see FIG. **1**) or of cross-pollination of F_1 hybrid plants, a second generation (S_1 or F_2) hybrid may be produced. Non-parental genetic combinations occur in these offspring due to independent assortment at meiosis of genes on different chromosomes and by recombination of genes on homologous (matched and paired at meiosis) chromosomes. Because of this further shuffling of the genetic material from the F_1 into the F_2, some of the F_2 hybrid plants produce less desirable plants than those of the F_1 in terms of the traits discussed above, due to homozygosity and other disruption of the F_1 genetic complement. In addition, there is increased variability overall of trait performance in the F_2 due to this extensive genetic shuffling, in particular, if many loci are involved in controlling a particular trait. It is not generally beneficial, therefore, for farmers to save the seed of F_1 hybrids. Rather a cycle of purchase by farmers each year of F_1 hybrid seed for planting is the rule. Corn breeders attempt to market new improved seed each season to attract these consumers.

North American farmers plant over 70 million acres of corn at the present time. There are extensive national and international programs in commercial corn breeding. Clearly this endeavor has a major impact on humanity in the form of food production. Basic methods of cross breeding inbred lines to produce hybrids are well known in concept by those skilled in the art. However, actual manipulation of these basic methods to generate improved hybrids is a delicate, arduous and sophisticated process. Breeders armed with methods to physically control plant breeding, and with an array of inbred lines with various known phenotypic traits, cannot expect to merely go into the field with these inbred

lines, breed them using well-established general methods, and walk out of their laboratories, greenhouses and fields with superior hybrids.

One of the first difficulties encountered is in breeding superior inbred parental lines, due to the difficulties discussed above which are inherent in inbreeding, for example, reduced vigor.

The skilled corn breeder also must make determinations regarding which combinations of these inbred lines should be selected to produce improved hybrids. None of the traits selected for commercial desirability are expressions of genes operating in a vacuum. Rather, to produce a plant which as a whole has an array of desirable characteristics, there must be a balance in terms of improvements. Phenotypic traits may show positive or negative correlations within inbred lines and between those lines and their hybrid progeny. Improving one trait may lead to poor outcome of another. Furthermore, hybrid plants that are beneficial in one set of environmental conditions may do poorly in others. With the increased need for increased food production within diverse areas of the world, and for transferring the growing of various crops to different locations of the world for maximum input and control of local persons over their agricultural destiny, it is important to develop wide ranges of hybrids that are going to perform well in both specific and general ecological and commercial niches.

Evidence of the difficulties inherent in commercial crop breeding is provided by the continual and highly competitive research in both the laboratory and the field revolving around improvement of inbred and hybrid lines. Removal of some of the uncertainty in large scale and expensive field testing is resulting from the application of methods of molecular biology whereby segments of the genetic complement may be singled out for faster, more selective and more successful breeding, and genetic complements may be combined in vitro, that is, in laboratory tissue culture vessels rather than in corn fields.

Some of the phenotypic traits for which improvements have continually been sought by hybridization of corn, include the production of varieties characterized by markedly improved yields, better stalks, better roots, and improved resistance to insecticides, pests and disease and markedly more uniform characteristics. With mechanical harvesting of many crops, uniformity of plant characteristics such as germination and stand establishment, growth rate, maturity, and fruit size, is important. Other desirable phenotypic characteristics for field crops include tolerance to heat and drought, reduced time to crop maturity, and better agronomic quality. However, despite some successes in breeding programs in the 1900's, progress is painstakingly slow—each qualitative improvement representing a small quantitative step.

Currently, it appears as if there is polygenic control over most commercially desirable traits such as yield. This means that many genes, generally on many chromosomes, contribute to the phenotypic appearance of the plant. The variance of the trait in inbred lines is less than that expected in hybrids formed from inbreds because of intralocus and interlocus interactions. Consequently, selective breeding programs to improve crops are not completely predictable.

SUMMARY OF THE INVENTION

Corn is the most important crop in the United States (Duvick, 1984). Consequently, a great deal of effort is expended to increase corn production and improve quality. A major method to achieve these goals is to produce hybrid plants which are superior to their inbred parents.

This invention addresses some of the shortcomings in the prior art of corn hybridization, and discloses a corn hybrid genetic complement that, when expressed in the form of a corn plant, exhibits superior characteristics that include increased yield. Genetic complement refers to that aggregate of nucleotide sequences that, when expressed in corn cells, yields a phenotype in corn plants, or components of plants including cultured cells, which includes phenotypic traits within specified quality and quantity ranges. As an example of a phenotypic trait, the improved yield of the hybrid of this invention compared to 3 commercially successful hybrids of similar maturity is dramatic, from between 5.1–8.3 bushels per acre. This is to be contrasted with the increase in U.S. corn yield of about 2 bushels per acre in recent times reported by Troyer (1990). This improvement was equated by Troyer to a value of about $330 million.

This invention relates to a hybrid genetic complement formed by the combination of a haploid genetic complement from each of the inbred lines of corn designated respectively, B73HT and MBUB. B73HT is a public line available through Iowa State University. MBUB is a proprietary line. The hybrid complement is designated DK671.

A deposit of 2500 seeds of inbred plants designated B73HT and MBUB was made with the American Type Culture Collection, Rockville Pike, Bethesda, Md. on Apr. 15, 1994. Those deposited seeds have been designated, for public accession purposes, as 75741 and 75739, respectively. These deposits were made in accordance with the terms and provisions of the Budapest Treaty relating to deposit of microorganisms.

The deposits are made for a term of at least thirty (30) years and at least five (05) years after the most recent request for the furnishing of a sample of the deposits was received by the depository. Prior to making those deposits, Applicant states that seeds of inbred corn plants B73HT and MBUB are on deposit at DeKalb Plant Genetics, Sycamore, Ill., and that such seeds are accessible and available to the Commissioner of Patents and Trademarks during pendency of this application.

One method used to produce the hybrid genetic complement was to combine the genetic complements of two different inbred lines. These lines were produced by repeated crossing of ancestrally related corn plants to concentrate certain genes within the inbred lines.

The hybrid genetic complement of the present invention was produced by interbreeding two corn lines which differ in their allelic constitutions at least at some loci. Alleles are conditions of genes that generally occupy the same locus, or position on a chromosome. Genes are sequences of nucleic acids, more specifically, DNA. Different alleles are characterized by different sequences of the DNA. These sequences are still capable of occurring at particular loci, although because of their sequence variation, they may be transcribed as mRNA in different nucleic acid sequences. Depending on the coding equivalency of the altered sequences, there may be changes in the translation into amino acid sequences in the gene products.

In an illustrative embodiment, the parental complements were contributed to the hybrid of this invention by transmission as haploid complements, that is, in the form of gametes, each gamete comprising one member of the pair of alleles at each locus. Because the parents are members of inbred lines, all haploid complements derived from the parent are expected to be essentially the same genetically, with the exception of, for example, mutations and the presence of heterozygous loci if there was not 100% homozygosity.

Because the inbred lines by definition are homozygous at most loci, the resulting hybrid is likely to be heterozygous at most loci unless the parental lines had the same allelic complement at some loci, perhaps due to common ancestry. The goal of the corn breeder for this invention was to produce heterosis, a phenomenon wherein the heterozygote produces a phenotype that is more desirable than that exhibited by either parent. In the hybrid genetic complement disclosed herein, that goal has been achieved. The complement designated DK671 shows superior traits when compared to other commercially successful hybrids of identical or equivalent maturity.

When expressed as a plant or its components thereof, the hybrid genetic complement has advantages when compared to other hybrid competitors of similar maturity. Maturity is a concept well known to those skilled in the art and refers to the observation that plants of different genotypes take different times to mature. This period must fit the temperature range of the environment in which the plants are raised to permit reproduction (FIG. 2). In other words, if a plant does not mature before the temperature drops below a permissible level, the plant can not be successful.

The hybrid corn plant designated DK 671 typically has a relative maturity of about 116 days. It is particularly suited for growth in irrigated areas in Colorado, Kansas and Nebraska, and performs well in growth environments conductive to high yields. It typically produces significantly higher yields, 5.1 to 8.3 bushels per acre, based on FACT trials. These performance and comparison trials are performed on actual farms under conditions which approximate the actual growing conditions used by farmers who will eventually purchase the seeds of the hybrid. These are "real life" trials to follow up and support research testing. Research testing is a more vigorous evaluation of basic characteristics of hybrids on smaller plots of land. Other favorable phenotypic characteristics of DK 671 comprise superior seedling vigor, stay green, stalks and roots (see Appendix for definitions of these traits). Superior stalk strength and uniformity of hybrid corn aid in the mechanical harvest and storage of the crop. Tables 1, 3–5 lists identifying features and characteristics of these hybrids. Table 2 compares their performance with other commercially successful hybrids. It is unexpected to improve several traits at once. "Gains may be made in the defensive traits but not in yield, or new hybrids may be improved in yield but not in defensive traits." (Duvick, 1984, p. 47).

Although the genetic complements of the inbred parental lines will be generally the same as that contained in haploid pollen or eggs, there may be some effects on offspring of maternal cytoplasmic factors. In addition, one of the parental lines may be preferred as the male and the other as the female due to phenotypic characteristics of the parental plant that affect reproduction. For example, one of the lines may have higher seed yield, one may shed pollen better, one may have preferred seed or tassel characteristics. For production of hybrid DK671, B73HT is preferred as the female, MBUB as the male parent.

In embodiments wherein the hybrid genetic complement is produced by field breeding schemes or by another method wherein gametes designated as pollen and egg are combined, the preferred source for the maternal haploid complement is B73HT and for the paternal haploid complement MBUB. MBUB is preferred as a paternal rather than maternal source because of poor seed quality from this line.

For other methods of combining the B73HT and MBUB genetic complements, for example, in vitro combinations performed in tissue culture, there need be no preferred maternal or paternal designation. Moreover, the genetic complements combined in vitro may represent only the subset of genes necessary to produce the superior phenotype of DK671. Nucleic acid segments that have no necessary coding function, need not be included.

The hybrid genetic complement designated DK671 exists in all somatic (non-germinal) cells of a corn plant, and is claimed in all those aspects, in particular, roots, stems, leaves, seeds and all flowering parts, including pollen grains. The pollen grains are haploid samples of the diploid genetic complement of the hybrid. These haploid samples result from independent segregation of the individual maize chromosomes and shuffling of the genes on homologous chromosomes by the natural recurring phenomenon of recombination at meiosis.

The hybrid genetic complement may be produced in several ways, either in vivo or in vitro. The following in vivo method comprises a breeding scheme which may be used to produce a hybrid corn plant in the field:

(a) planting in pollinating proximity seeds from inbred corn lines having the designations, B73HT and MBUB;

(b) cultivating corn plants resulting from the planting before the time of flowering;

(c) emasculating the plants of inbred corn line B73HT;

(d) allowing cross-pollination to occur between said corn lines; and

(e) harvesting seeds produced by the plants of the emasculated corn plants from the line designated B73HT.

Hybrid plants may be grown from seeds with the genetic complement disclosed herein by methods well known to those skilled in the art.

To produce the hybrid genetic complement in vitro, a plant may be regenerated from cells in culture (Gordon-Kamm, et al., 1990). To regenerate hybrid plants, cells are obtained which comprise the hybrid genetic complement, for example, somatic cells from a DK671 corn plant. These cells are then cultured in vitro in a media comprising an embryogenic promoting hormone until callus organization is observed. At this point, cells are transferred to media which includes a tissue organization promoting hormone. After tissue organization is observed, the cells are subcultured onto media without said hormone, to allow for shoot elongation or root development. Finally, the plantlets are transferred onto a minimal medium to provide for hardening of the plant.

Embodiments of the embryogenic promoting medium are dicamba, 2,4-D and the like. Embodiments of the tissue organization promoting medium comprises BAP, myoinositol, 2,4-D, ABA, NAA, IAA and 2IP.

IBA may be used to stimulate rooting. Minimal media comprises Clark's media.

The combination of genetic complements of the inbred lines B73HT and MBUB to yield the hybrid complement disclosed herein is claimed within the scope of this invention regardless of the method used to produce it.

All parts of plants derived from the hybrid DK671 genetic complement are claimed as part of the present invention including roots, stems, leaves and all flowering parts including pollen grains, because all have the novel genetic complement of this invention.

This invention also relates to the hybrid genetic complement contained in seeds. Plants grown from these seeds by methods well known to those skilled in the art are expected to exhibit the characteristics listed in Tables 1 through 5.

Phenotypic traits characteristic of the expression of the hybrid genetic complement of this invention include those that are distinguishable by electrophoretic separation of DNA sequences cleaved by various restriction endonucleases. These traits (genetic markers) are termed RFLP (restriction fragment length polymorphisms).

Restriction fragment length polymorphisms (RFLPs) are genetic differences detectable by DNA fragment lengths, typically revealed by agarose gel electrophoresis, after restriction endonuclease digestion of DNA. There are large numbers of restriction endonucleases available, characterized by their nucleotide cleavage sites and their source, e.g., the bacteria *E. coli.* Variations in RFLP's result from nucleotide base pair differences which alter the cleavage sites of the restriction endonucleases, yielding different sized fragments.

Restriction fragment length polymorphism analysis was conducted by Native Plants Incorporated (NPI) (Table 4). This service is available to the public on a contractual basis. For this analysis, the genetic marker profile of the parental inbred lines were determined. Because these inbreds are essentially homozygous at all relevant loci, they should have only one allele at each locus. Consequently, the diploid genetic marker profile of the hybrid offspring of the inbred parents should be the sum of those parents, e.g., if one parent had the allele A at a particular locus, and the other parent had B, the hybrid AB is by inference. The RFLP complement is presented in Table 4.

Probes were prepared to the fragment sequences, these probes being complementary to the sequences thereby being capable of hybridizing to them under appropriate conditions well known to those skilled in the art. These probes are labelled with radioactive isotopes or fluorescent dyes for ease of detection. After the fragments are separated by size, they were identified by the probes. Hybridization with a unique cloned sequence permits the identification of a specific chromosomal region (locus). Because all alleles at a locus are detectable, RFLP's are codominant alleles, thereby satisfying a criteria for a genetic marker. They differ from some other types of markers, e.g, from isozymes, in that they reflect the primary DNA sequence, they are not products of transcription or translation. Furthermore, different RFLP profiles result from different arrays of restriction endonucleases.

Other characteristic phenotypic traits include the isozyme variants listed in Table 5. These are codominant genetic markers that delineate segments of nucleic acids characterizing the genetic complement.

An important use of genetic markers is to reconstruct ("reverse engineer") the parental genetic complements of an offspring. For example, the hybrid genetic complement results from the combination of two inbred parental complements. The genetic complements of the parental lines may be determined by comparing the RFLP and isozyme genetic profiles of the hybrid and that of B73HT, a public line. The remaining complement is that of MBUB. The markers are inherited in codominant fashion and follow well-known rules of mendelian inheritance. That means if a hybrid has marker AB, and one of the parental lines has marker A (genotype AA), the other parent by inference has marker B. If the parent is an inbred line, it is expected to be BB, because it is essentially homozygous. The marker genetic complement of DK671 is presented for RFLP and isozymes in Tables 4 and 5. The genetic complement of parental line B73HT, a publicly available line, may be determined by following the marker detection methods disclosed within. The parental contribution of MBUB can be determined by inference. Because B73HT and MBUB are inbred lines, marker profiles are not disrupted by linkage disequilibrium, and are likely to remain associated with adjacent genetic segments.

DEFINITIONS

Cross-pollination—pollen from a flower of one plant is used to fertilize a different maternal plant.

Elite Germplasm—in its area of adaptation, germplasm that is considered by breeders to possess a high level of favorable traits.

Substantially Similar Genetic Complement—genetic complements having nucleic acid sequences which code for the same introns or mutant or variant sequences which either are codon equivalents or do not affect the phenotype as disclosed herein.

Plant Components—all somatic cells, including protoplasts, calli, and parts of plants, from which plants can be regenerated in tissue culture, e.g., flowers, kernels, ears, cobs, leaves, husks, and stalks.

Self-pollination—pollen from one flower is transferred to the same or another flower of the same plant.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects and advantages of the invention will become apparent upon reading the following detailed description and upon reference to the drawings in which:

FIG. **1** is a diagram of the life cycle of maize, *Zea mays.*

FIG. **2** is a relative maturity map used to define temperature ranges for corn plant growth.

While the invention is susceptible to various modifications and alternative forms, a specific embodiment thereof has been shown by way of examples and will herein be described in detail. It should be understood, however, that it is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention relates to a hybrid genetic complement produced by combining the haploid genetic complements of two parental inbred lines of corn.

Corn has a diploid phase which means two conditions of a gene (two alleles) occupy each locus (position on a chromosome). If the alleles are the same at a locus, there is said to be homozygosity. If they are different, there is said to be heterozygosity. In a completely inbred line, all loci are homozygous. Because many loci when homozygous are deleterious to the plant, in particular leading to reduced vigor, production of inbred lines is an unpredictable and arduous process. Under some conditions, heterozygous advantage at some loci effectively bars perpetuation of homozygosity.

These parental lines have been bred to increase their homozygosity. Based on well-established genetic principles, it is known that a homozygous line crossed to another homozygous line is expected to yield hybrids, usually designated F_1, that are genotypically the same barring rare events such as mutation. Of course, the phenotype of the hybrid organism may not always be identical when the same inbred lines are used as parents because the comparability of the phenotypic expression of the genotype depends on the similarity of the environmental conditions interacting with the genotype to produce the phenotype. In general, the phenotypes are expected to be similar unless the phenotype or aspects thereof is strongly dependent on environmental conditions.

The development of corn hybrids to generate crops with improvements in commercially desirable traits, is a sequential and laborious process of recurrent selection of inbred or partially inbred lines to form breeding pools from which new lines are further selected either by self-pollination and selection of desired phenotypes, and further inbreeding. Inbreds are then selected to be crossed with other inbreds to form hybrids which are evaluated in terms of their commercial potential. Many generations of breeding are involved before final selections are made of desirable hybrids for commercial use. "Uncommonly great genetic variability is present in and among the diverse lines, varieties, and races of corn in the world." (Coe et al., 1988, p. 111.) A breeders task is to delve into the morass of genetic variability and extract a new, improved combination of genes.

Herculean efforts have been undertaken, particularly in the 1900's by commercial seed producers in continuous research and development directed toward crop improvement. Selection and development of inbred lines is tenuous and tedious. Challenges posed by varying climates, new pests, and economic trends, prevent successful selection of "off the shelf" inbred lines to get a hybrid with desired traits. Troyer (1990), chronicles the excruciatingly tedious, complex venture of corn breeding for crop improvement. Although there are some well-established corn breeding techniques, they are not cook-book protocols which can be implemented without significant human interaction. Moreover, they are guidelines for improvement, not blueprints for success.

Sprague and Eberhart (1977) illustrates the complexity of the involvement of the human breeder in corn programs. More importantly, Sprague discusses the relevance of additive and non-additive genetic effects. In additive effects, the genes contributed to an offspring by a certain line produce characteristic effects, usually regardless of the order of pairing, when combined with genetically analogous germplasm. Non-additive effects, however, must be considered in predicting phenotypic effects resulting from crossing certain genotypes. Dominance (intra-locus genetic interactions) and epistasis (inter-locus effects) are not completely predictable functions of the simple combinations of parental traits.

It is not expected that a parental line with superior traits will convey these traits to offspring of a cross with another parental line. Transmission of traits depends on the nature of the genetic transmission and the basis for the phenotypic trait. Those traits due to recessive inheritance in either parent, are expected to disappear or be modified in a hybrid because the homozygosity underlying the trait is disrupted unless identical alleles are donated from each parent to the relevant loci in the hybrid. The goal is that the disruption is beneficial, that is, heterosis results. For any one cross that turns out to be superior, many, many such crosses will likely fail. Interactions among loci (epistasis) are also generally unpredictable in offspring of new genetic parental combinations.

The performance of an inbred line is judged by its performance when crossed with other inbred lines to produce hybrids.

The general combining ability of a particular inbred is determined by its average performance in a series of hybrid combinations. General combining ability is said to measure mainly additive genetic effects. Specific combining ability (sca) refers to the performance of two particular inbreds in a specific cross. Specific combining ability measures nonadditive types of gene interactions.

One of the traits for which hybridization has been employed to cause improvement is yield per acre of corn. For example, in the April 17, 1974 edition of the Wall Street Journal, an article entitled "In Search of Superbean," it was pointed out that soybeans could not easily be hybridized and therefore fell far behind corn and productivity increased. During the period of 1950 to 1973, soybeans increased in productivity from 21.8 to 27.8 bushels per acre, whereas corn increased from 38.4 to 91.4 bushels per acre. Improvements since that time have been sought by sophisticated plant and genetic manipulation.

Whether two particular inbreds combine to produce a high-yielding single cross depends upon the extent to which favorable genes for yield from one inbred supplement those contributed by the second inbred. The hybrid genetic complement resulting from combining B73HT with MBUB has produced an unexpectedly high-yielding hybrid line also with other desirable traits.

Some of the other desirable traits for DK671 are also important commercially. Commercial hybrid maize generally grows from about 7–9 feet tall with each plant having either one or two ears. The ear normally grows about one third the way up the plant or about $2\frac{1}{2}$ to $3\frac{1}{2}$ feet from the ground. Consequently, the maize plant, while providing a large ear has a substantial leaf and stalk structure and a considerable mechanical stability problem in that the heavy ear is about 3 feet from the ground with 6 feet of stalk and tassels extending above that. In the past, efforts have been made to develop strong stalk and branching the secondary roots in maize to help alleviate the stability problem. While these efforts have improved the mechanical stability of maize considerably, heavy windstorms and rain can still wreck havoc in a field of maize. Excellent plant seedling vigor is advantageous as well as germination, early stand and stay green characteristics. DK671 shows significant improvements in these traits.

Mixing parental germplasm from inbred lines in the cauldron of the F_1 hybrid does not lead to a completely

predictable spectrum of traits in that hybrid. This is because traits in the hybrid result from interactions among genes at the same locus (dominance, overdominance), genes at different loci (epistasis) and genes and environments. The meiotic shuffling of the genes plus the interaction of genes with the environment will lead to the trait. It is the trait which is sought as commercially desirable.

Hybrids may be produced from single or multiple parental inbred line crosses, although of course only two parents may be used to produce any particular plant. However, successive single cross hybrids from different inbred lines will lead to the merging of the genetic parental complements of various inbred lines into one hybrid. It is this matrix of multiple merging that illustrates the sophisticated methods of selection and crop improvement.

Complex mathematical models have been developed to predict offspring phenotypes based on parental genotypes and the environment (Falconer, 1960; Sprague and Eberhart, 1977).

Breeding Methods to Develop Hybrids

Reciprocal crosses refer to interchange of the sex of the parents. When the two inbred parental lines B73HT and MBUB were crossed to yield the hybrid DK671, either parent may serve as the maternal or paternal plant. For many crosses, the outcome is the same regardless of the assigned sex of the parental lines. However, there is often one of the parental lines that is preferred as the maternal line because of increased seed yield and production characteristics. Some lines produce tighter ear husks leading to more loss, for example due to rot. There may be delays in silk formation which deleteriously affect timing of the reproductive cycle for a pair of parental inbreds. Seed coat characteristics may be preferable in one line. Pollen may be shed better by one line. Other variables may also affect preferred sexual assignment of a particular cross. In the production of DK671, B73HT is preferred as the female, MBUB as the male, parent.

The goal of the corn breeding program disclosed herein was to manipulate the genetic complement of corn to generate new combinations of genes which interacted to yield new or improved traits (phenotypic characteristics).

The general process began with the production of inbred lines. These lines were produced by repeated crossing of ancestrally related corn plants to try and concentrate certain genes within the inbred lines. Corn has a diploid phase which means two conditions of a gene (two alleles) occupy each locus (position on a chromosome). If the alleles are the same at a locus, there is said to be homozygosity. If they are different, there is said to be heterozygosity. In a completely inbred line, all loci are homozygous. Because many loci when homozygous are deleterious to the plant, in particular leading to reduced vigor, less kernels, weak and/or poor growth, production of inbred lines is an unpredictable and arduous process. Under some conditions, heterozygous advantage at some loci effectively bars perpetuation of homozygosity.

Inbreeding requires coddling and sophisticated manipulation by human breeders. Even in the extremely unlikely event inbreeding rather than crossbreeding occurred in natural corn, achievement of complete inbreeding cannot be expected in nature due to well known deleterious effects of homozygosity and the large number of generations the plant would have to breed in isolation. The reason for the breeder to create inbred lines is to have a known reservoir of genes whose gametic transmission is at least somewhat predictable. The inbred lines used in this invention were B73HT, publicly available from Iowa State University. MBUB is a proprietary line.

The development of inbred lines generally requires at least about 5 to 7 generations of selfing. Inbred lines are then cross-bred in an attempt to develop improved F_1 hybrids. Because there is no adequately reliable way to predict the overall phenotype of any particular F_1 hybrid, tens of thousands of hybrids resulting from large numbers of crosses are required and must be evaluted each year. These hybrids are then screened and evaluated in small scale field trials. Typically, about 10–15 phenotypic traits are measured. These traits are selected for their potential commercial value. A selection index of the most commercially important traits is used to help evaluate hybrids. For DK671 yield, per cent moisture, stalk lodging, root lodging, and dropped ears were used in the selection index. FACT, an acronym for Field Analysis Comparison Trial (strip trials), is an on-farm testing program employed by DeKalb Plant Genetics to perform the final evaluation of the commercial potential of a product.

After one year of evaluation, approximately 95% of the hybrids are rejected on the basis of their performance not being above that of previously developed hybrids.

During the next several years, a progressive elimination of hybrids occurs based on more detailed evaluation of their phenotype. Eventually, strip trials (FACT) are conducted to formally compare the experimental hybrids being developed with other hybrids, some of which were previously developed and generally are commercially successful. That is, comparisons of the experimental hybrids were made to competitive hybrids in order to determine if there was any advantage to further commercial development of the experimental hybrids. Comparisons were made to hybrids of similar or identical maturity.

Time to maturity is an important characteristic for a corn line and must match the environment in which the corn is to be planted. If there is only enough heat accumulated to mature a hybrid corn plant in 80 days, and the plant requires 125 days, there will not be time enough to enter a reproductive phase. Maturity of a hybrid is not completely predictable from that of the parental inbreds. Another complication is that the range of maturity will be based on the selection environments.

Strip trials compare the phenotypes of hybrids grown in as many environments as possible. Strip tests were performed in many environments to assess overall performance of the new hybrids and to select optimum growing conditions. The corn being compared is of similar maturity. The corn is grown in close proximity, consequently, environmental factors that affect gene expression are minimized. These factors include moisture, temperature, sunlight and pests.

For a decision to be made that a hybrid is worth making commercially available, it is not necessary that the hybrid be better than all other hybrids. Rather, significant improvements must be shown in at least some traits that would create improvements in some niches.

DK671 was found to be an unexpectedly superior hybrid. Results of experiments designed to test response

of DK671 to insects and resistance to disease, are shown in Table 1. The levels of disease and insect resistance make DK671 a highly acceptable hybrid in its marketplace.

cessful hybrids designated DK656, DK677, DK689 and PION3377. The increased yield in bushels per acre is 5.1 to 8.3. Significant improvements are also shown in Table 2 for many other traits, notably superior seedling

TABLE 1

REACTION OF DK671 TO DISEASE AND INSECTS

	FOLIAR DISEASES[1]									SYSTEMIC[2]		INSECT[3]				HARVEST[4]		
YEARS	HM	HT1	HTG	HT2	HC2	HC3	CG	CZ	CN	CLN	SR	ON1	ON2	DGG	DSP	ER	STR	LSS
1987	3					6	2											3
1988	4	1	+	1		6						7	9	94				2
1989	5	1	+	3	1	7	2	8	1	7	25		6			3	6	4
AVG.	4	1		2	1	6	2	8	1	7	25	7	8	94		3	6	3

CODE	=	DESCRIPTION
[1]FOLIAR DISEASES (FUNGAL)		Reactions to various pathogens. Numerical ratings for foliar diseases times 10 is approximately equal to percent total plant infection.
Hm	=	*Helminthosporium maydis* race 0 rating.
Htl	=	*Helminthosporium turcicum* race 1 rating.
HtG	=	- = Presence of a Ht chlorotic-lesion type resistance. - = Absence of a Ht chlorotic-lesion type resistance. -- = Segregation of a Ht chlorotic-lesion type resistance.
Ht2	=	*Helminthosporium turcicum* race 2 rating.
Hc2	=	*HelminthosRorium carbonum* race 2 rating.
Hc3	=	*Helminthosporium carbonum* race 3 rating.
Cg	=	*Colletotrichum graminicola* rating. *Following Cg rating indicates premature death of that entry to *C. graminicola*.
Kz	=	*Kabatiella zeae* rating.
Cz	=	*Cercospora zeae-maydis* rating.
Cn	=	*Corynebacterium nebraskense* rating.
[2]SYSTEMIC DISEASES (VIRAL)		Reactions to various systemic diseases.
CLN	=	Corn Lethal Necrosis (combination of Maize Chlorotic Mottle Virus and Maize Dwarf Mosaic Virus) rating: numerical ratings are based on a severity scale where 1 = most resistant to 9 = susceptible.
SVC	=	Southeastern Virus Complex (combination of Maize Chlorotic Dwarf Virus and Maize Dwarf Mosaic Virus) rating; numerical ratings are based on a severity scale where 1 = most resistant to 9 = susceptible (1988 reactions are largely Maize Dwarf Mosaic Virus reactions).
Sr	=	*Sphacelotheca reiliana* rating is actual percent infection.
[3]INSECTS		
On1	=	*Ostrinia nubilalis* 1st brood rating (1 = resistant to 9 = susceptible).
On2	=	*Ostrinia nubilalis* 2nd brood rating (1 = resistant to 9 = susceptible).
Dgg	=	*Diatraea grandiosella* girdling rating (values are percent plants girdled and stalk lodged).
Dsp	=	*Diabrotica* species root ratings (1 = least affected to 9 = severe pruning).
[4]HARVEST DISEASES		
ER	=	Ear rot rating (values approximate percent ear rotted).
STR	=	Stalk rot rating (values represent severity rating of 1 ≦ 25% of inoculated internode rotted to 9 = entire stalk rotted and collapsed).
LSS	=	Late season standability (values times 10 approximate percent plants lodged in disease evaluation plots).

Table 2 presents examples of comparisons of performance data for DK671 versus selected hybrids of commercial value. These data represent results across years and locations for strip trials.

The "NTEST" represents the number of paired observations in designated tests at locations around the United States.

As can be seen in Table 2, DK671 has significantly higher yield when compared to four commercially successful hybrids designated DK656, DK677, DK689 and PION3377. The increased yield in bushels per acre is 5.1 to 8.3. Significant improvements are also shown in Table 2 for many other traits, notably superior seedling vigor, stay green, stalks, and roots. Seedling vigor refers to rates of early development of plants in the spring on a 1–9 scale (9=best). Stay green is a scale 1–9 (9=best) of general health of the plant after the ear leaves have reached maturity. Stalk and root lodging are measures of standability. Stalk lodging is measured as the percent of plants broken over below the ear at harvest. Root lodging is the percent of plants leaning at greater than a 30° angle to the ground.

TABLE 2

COMPARISON OF DK671 WITH 4 COMMERCIALLY SUCCESSFUL HYBRIDS

	NTEST	SI %	YIELD	MOIST	SV	FGDU	PHT	EHT	BAR	SG	D %	STL	RTL	TWT	ESTR
DK671	R 205	106.3	158.5	20.0	6.1	1380	95.9	46.6	1.5	3.8	0.1	3.5	1.5	53.5	115.3
DK656		99.2	151.9	19.5	5.5	1413	93.8	49.4	0.9	2.9	0.1	4.9	2.1	56.0	114.5
		**	**	**	**	**	**	**		**		**	*	**	
DK671	F 87	104.8	157.3	20.9							0.2	2.6	2.8	54.3	116.5
DK656		98.7	150.9	19.8							0.4	4.1	3.9	56.8	114.3
		**	**	**							*	**	+	**	
DK671	R 203	106.3	157.8	20.2	6.1	1381	95.9	46.6	1.5	3.8	0.1	4.1	1.5	53.5	115.2
DK677		102.6	155.4	20.9	5.9	1418	94.2	43.7	3.0	5.4	0.1	4.5	1.1	56.8	115.8
		**	*	**		**	*	**		**				**	

TABLE 2-continued

		COMPARISON OF DK671 WITH 4 COMMERCIALLY SUCCESSFUL HYBRIDS													
	NTEST	SI %	YIELD	MOIST	SV	FGDU	PHT	EHT	BAR	SG	D %	STL	RTL	TWT	ESTR
DK671	F 74	105.2	159.4	20.5							0.0	2.1	1.4	54.5	116.6
DK677		101.1	154.3	20.4							0.1	2.3	0.4	57.2	116.1
		*	**											**	
DK671	R 78	110.1	155.2	19.4	6.5	1433	93.2	45.8	1.5	4.3	0.2	2.3	2.1	54.0	115.8
DK689		91.3	141.8	19.8	6.6	1505	92.0	49.0	3.1	5.4	0.4	5.4	4.5	55.3	116.6
		**	**	*		**		*		+	+	**	*	+	
DK671	F 43	101.9	145.4	20.2							0.1	3.2	2.7	54.5	116.8
DK689		92.6	137.1	21.2							0.4	3.0	1.3	55.9	118.4
		**	**	**							+			**	
DK671	R 201	106.3	157.9	20.1	6.1	1380	95.9	46.6	1.5	3.8	0.1	3.5	1.5	53.5	115.3
PION3377		101.1	151.7	17.9	5.8	1389	93.9	47.3	1.9	3.3	0.1	6.8	3.0	55.6	112.6
		**	**	**	*	+	**			+		*8	**	**	
DK671	F 16	104.0	164.3	18.9							0.3	3.4	2.0	54.4	114.8
PION3377		97.4	158.5	17.5							0.5	7.4	2.4	56.6	111.7
		**	*	**								*		**	

Where
R = research test results
F = FACT testing
SI % = Selection index, see 67 in appendix.
Yield = see 68 in appendix.
Moist = see 69 in appendix.
SV = seedling vigor, a 1-9 rating of early season plant growth wher 9 = best.
FGDU = energy required to flower (see 66 in this appendix).
PHT = see 6 in appendix.
EHT = see 41 in appendix.
BAR = % barren, % of plants that lack an ear.
SG = see 70 in appendix.
D % = see 71 in appendix.
STL = % of platists broken over below the ear at harvest.
RTL = % of plants leaning at greater than 30° angle to the ground.
TWT = see 65 in appendix.
ESTR = estimate of relative maturity (days)
and significance levels are indicated as
+ = 10%
* = 5%
** = 1%

A general description of the DK671 hybrid is presented in Table 3.

TABLE 3

MORPHOLOGICAL CHARACTERISTICS OF THE DK671 PHENOTYPE
(See Appendix)

CHARACTERISTIC	DK671 VALUE*
1. Seedling	
Percent Emergence	97
Height cm.	30.0
Color	Dark Green
Vigor 1-5 Rating	2
2. Stalk	
Plant Height cm.	300.3
Ear Height cm.	138.7
Diameter (Width) cm.	2.8
Anthocyanin	Absent
Nodes With Brace Roots	1.8
Brace Root Color	Green
Internode Direction	Straight
Internode Length cm.	18.8
3. Leaf	
Angle	Upright
Number	20.7
Post Poll Color	Dark Green
Length cm.	87.7
Width cm.	12.1
Sheath Anthocyanin	Weak
Sheath Pubescence	Medium
Marginal Waves	Few
Longitudinal Creases	Absent
4. Tassel	
Total Length cm.	37.0
Spike Length cm.	29.6
Peduncle Length cm.	11.1
Attitude	Open
Branch Angle	Intermediate
Branch Number	8.6
Anther Color	Yellow
Glume Color	Green
Glume Band	Absent

TABLE 3-continued

MORPHOLOGICAL CHARACTERISTICS OF THE DK671 PHENOTYPE
(See Appendix)

CHARACTERISTIC	DK671 VALUE*
5. Ear	
Silk Color	Yellow
Number Per Stalk	1.1
Position (Attitude)	Upright
Length cm.	19.1
Shape	Semi Conical
Diameter cm.	5.1
Weight gm.	294.3
Shank Length cm.	15.2
Shank Internode Number	9.4
Husk Bract	Short
Husk Cover cm.	2.4
Husk Opening	Intermediate
Husk Color Fresh	Light Green
Husk Color Dry	Buff
Cob Diameter cm.	3.0
Cob Color	Pink
Cob Strength	Strong
Shelling Percent	87.0
6. Kernel	
Row Number	18.6
Number Per Row	46.0
Row Direction	Slightly Curved
Type	Dent
Cap Color	Yellow
Side Color	Orange
Length (Depth) mm.	14.4
Width mm.	8.3
Thickness	4.3
Weight of 1000K gm.	353
Endosperm Type	Normal
Endosperm Color	Yellow
7. Other	
Uniformity 1-5 Rating	1
GDUS to 50% Pollen Shed	1384

TABLE 3-continued

MORPHOLOGICAL CHARACTERISTICS OF THE DK671 PHENOTYPE (See Appendix)	
CHARACTERISTIC	DK671 VALUE*
GDUS to 50% Silking	1415

*These are typical values of DK671 plants. Values may vary due to environment. Other values that are substantially equivalent are also within the scope of the invention.

Genetic Markers to Identify Plants

Markers are genes, the phenotypic expressions of which are used to identify the presence of other genes or genetic complements which cosegregate with the markers through meiosis and appear jointly in offspring. Markers are generally codominant, that is, both alleles at a marker locus are readily detectable in a heterozygote. Markers which are useful in plant breeding comprise isozymes and restriction fragment length polymorphism (RFLP's). RFLP analysis has been performed on the parents of DK671.

Isozymes are forms of proteins that are distinguishable, for example, on starch gel electrophoresis, usually by charge and/or molecular weight. The isozyme profile of DK671 is shown in Table 5.

A standard set of loci may be used as a reference set. Comparative analysis of these loci may be used to compare the purity of hybrid seeds, to assess the increased variability in hybrids compared to inbreds, and to determine the identity of seeds, plants, and plant parts. In this respect, an isozyme or RFLP reference set may be partially used to develop genotypic "fingerprints."

Table 5 lists the identifying numbers of the alleles segregating at isozyme loci types for hybrid DK671. This set of alleles are one set of identifiers for the genetic complements of this hybrid. These alleles are not known to be directly related to the desirable traits of these hybrid plants (Goodman and Stuber, 1980), but are useful to identify genomes.

TABLE 5

ISOZYME GENOTYPE FOR HYBRID DK671	
LOCUS	ISOZYME ALLELES
Acph	Seg*
Adh	4
Amp-1	4
Cat	9
Dia-1	8
Dia-2	4
Enp	6
Got-1	4
Got-2	4
Got-3	4
B-Glu	6/7
Hex-2	2
Idh-1	4/6
Idh-2	4/6
Mdh-1	6
Mdh-2	3.5/6
Mdh-3	16
Mdh-4	12
Mdh-5	12
Pgm-1	9
Pgm-2	4
6-Pgd-1	2/3.8
6-Pgd-2	5
Phi	4
Tpi-1	4
Tpi-2	4
Tpi-3	4
Tpi-4	4

TABLE 5-continued

ISOZYME GENOTYPE FOR HYBRID DK671	
LOCUS	ISOZYME ALLELES
#Plants Assayed	6

*Alleles segregating are 2/2; 2/4; one inbred line was not pure for that locus.

Methods for In Vitro Hybrid plant Regeneration

Hybrid plants can be grown from hybrid seeds by methods well known to those skilled in the art. Hybrid plants may also be regenerated from tissues of hybrid plants by use of in vitro laboratory methods of tissue culture.

In certain embodiments, recipient cells are selected following growth in culture. Where employed, cultured cells are preferably grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients are provided to the cells in the form of media, and environmental conditions are controlled. There are many types of tissue culture media comprising amino acids, salts, sugars, hormones and vitamins. Most of the media employed to regenerate hybrid plants will have some similar components, the media differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells have been previously described. Examples of these media include, but are not limited to the N6 medium described by Chu, et al. (1975) and the MS media, Murashige and Skoog (1962). In an exemplary embodiment for preparation of recipient cells, modifications of these media are available. A preferred hormone for such purposes is dicamba or 2,4-D. However, other hormones may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Modifications of these and other basic media may facilitate growth of recipient cells at specific developmental stages.

An exemplary embodiment for culturing recipient corn cells in suspension cultures includes using embryogenic cells in Type II (Armstrong and Green, 1985; Gordon-Kamm, et al., 1990) callus, selecting for small (10–30μ) isodiametric, cytoplasmically dense cells, growing the cells in suspension cultures with hormone containing media, subculturing into a progression of media to facilitate development of shoots and roots, and finally, hardening the plant and readying it metabolically for growth in soil.

Meristematic cells (i.e., plant cells capable of continual cell division and characterized by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, etc.) may be cultured.

EXAMPLES

Example 1

Methods of Producing Hybrid Genetic Complements: Breeding

Corn plants (*Zea mays* L.) can be bred by either self-pollination or cross-pollination techniques. Corn has male flowers, located on the tassel, and female flowers, located on the ear, on the same plant. Natural pollination occurs in corn when wind blows pollen from the

tassels to the silks that protrude from the tops of the incipient ears. Mechanical pollination may be effected either by controlling the types of pollen that can blow onto the silks or by pollinating by hand.

Two inbred lines were selected as parents, one of which is the inbred line B73HT, the other MBUB. B73HT is a publicly available line (Iowa State University). MBUB is proprietary. These parental lines were planted in pollinating proximity to each other. This may be achieved by planting the parental lines in alternating rows, in blocks or in any other convenient planting pattern. The plants of both lines were allowed to grow until the time of flowering. Advantageously, during this growth stage, the plants are in general treated with fertilizer and/or other agricultural chemicals as considered appropriate by the grower.

At the time of flowering, in the event that inbred line MBUB is employed as the male parent, the tassels of the other parental line, B73HT are removed from all plants employed as the female parental line. The detasseling can be achieved manually but also can be done by machine if desired.

The lines are then allowed to continue to grow and natural cross-pollination occurs as a result of the action of wind which is normal in the pollination of grasses, including corn. As a result of the emasculation of the female parent line, all the pollen from the male parent line, e.g., MBUB, is available for pollination because tassels, and thereby pollen bearing flowering parts, have been previously removed from all plants of the inbred line being used as the female in the hybridization. Of course, during this hybridization procedure, the parental varieties are grown such that they are isolated from other corn fields to prevent any accidental contamination of pollen from foreign sources. These isolation techniques are well within the skill of those skilled in this art.

Both of the parent inbred lines of corn are allowed to continue to grow until maturity, but only the ears from the female inbred parental lines are harvested to obtain seeds of a novel F_1 hybrid corn. If desired, corn produced in the male parent variety can be harvested, e.g., for grain use, but these seeds are not useful as hybrid seeds.

The novel F_1 hybrid seed produced may then be planted in a subsequent growing season with the desirable characteristics in terms of F_1 hybrid corn plants providing improved grain yields and the other desirable characteristics disclosed herein, being achieved.

Example 2

Methods of Producing Hybrid Genetic Complements: In Vitro Regeneration

Embryogenic calli are produced (Gordon-Kamm et al., 1990). Specifically, plants from hybrids produced from crossing the inbreds (B73HT×MBUB) are grown to flowering in a greenhouse. Explants from at least one of the following F_1 tissues: immature tassel tissue, intercalary meristems and leaf bases, apical meristems, and immature ears are placed in an initiation medium which contain MS salts, supplemented with thiamine, agar, and sucrose. Cultures are incubated in the dark at about 23° C. All culture manipulations and selections are performed with the aid of a dissecting microscope.

After about 5–7 days, cellular outgrowths are observed from the surface of the explants. After about 7–21 days, the outgrowths are subcultured by placing into fresh medium of the same composition. Some of the intact explants are placed on fresh medium.

Several subcultures later (after about 2 to 3 months) enough material is present from explants for subdivision of these embryogenic calli into two or more pieces. Callus pieces from different explants are not mixed. After further growth and subculture (about 6 months after embryogenic callus initiation), there are usually between 1 and 100 pieces derived ultimately from each selected explant. During this time of culture expansion, a characteristic embryogenic culture morphology develops as a result of careful selection at each subculture. Any organized structures resembling roots or root primordia are discarded. Any material known from experience to lack the capacity for sustained growth is also discarded (translucent, watery, embryogenic structures). Structures with a firm consistency resembling at least in part the scutellum of the in vivo embryo are selected.

The callus is maintained on agar-solidified MS-type media. The hormone used is 2,4-D. Visual selection of embryo-like structures is done to obtain subcultures. Transfer of material other than that displaying embryogenic morphology results in loss of the ability to recover whole plants from the callus.

Some calli will exhibit somaclonal variation. These are phenotypic changes appearing in culture.

Cell suspensions are prepared from the calli by selecting cell populations that appear homogeneous macroscopically. A portion of the friable, rapidly growing embryogenic calli is inoculated into MS Medium. The calli in medium are incubated at about 27° C. on a gyrotary shaker in the dark or in the presence of low light. The resultant suspension culture is transferred about once every seven days by taking about 5 to 10 ml of the culture and introducing this inoculum into fresh medium of the composition listed above.

For regeneration, embryos which appear on the callus surface are selected and regenerated into whole plants by transferring the embryogenic structures into a sequence of solidified media which include decreasing concentrations of 2,4-D or other auxins. Other hormones which may be used in the media include dicamba, NAA, ABA, BAP, and 2-NCA. The reduction is relative to the concentration used in culture maintenance media. Plantlets are regenerated from these embryos by transfer to a hormone-free medium, subsequently transferred to soil, and grown to maturity.

Progeny are produced by taking pollen and selfing, backcrossing or sibbing regenerated plants by methods well known to those skilled in the arts. Seeds are collected from the regenerated plants.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and herein be described in detail. It should be understood, however, that it is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

APPENDIX:

PLANT TRAIT DESCRIPTIONS

2. Seedling Emergence—Number of emerged plants divided by number of seeds planted, expressed as a percentage.
3. Seedling Height—Plant height at the 6–8 leaf stage in centimeters.
4. Seedling color—color of leaves at the 6–8 leaf stage (light green, dark green, anthocyanin).
5. Seedling vigor—Rated on a scale of 1–5 (1=best) at the 2–4 leaf stage.
6. Plant Height—Measured from soil to the tip of the tassel in centimeters.
7. Stalk Diameter—Diameter of the lowest visible internode, measured in centimeters.
8. Stalk Anthrocyanin—Rated 1–2 weeks after pollination as absent, basal-weak, basal-strong, weak, strong.
9. Stalk Nodes with Brace Roots—Average number of nodes having brace roots on each plant.
10. Stalk Brace Root Color—Color of brace roots observed 1–2 weeks after pollination (green, red, or purple).
11. Stalk Internode Length—Length of the internode above the primary ear in centimeters.
12. Stalk Internode Direction—Observed after pollination, straight or zig-zag.
13. Leaf Angle—Angle of the upper leaves to the stalk; 0°–30° =upright, 30°–60° =intermediate, 60°–90° =lax.
14. Leaf Number—Total number of leaves from the cotyledon leaf to the flag leaf.
15. Leaf Length—Length of the primary ear leaf measured in centimeters.
16. Leaf Width—Width of the primary ear leaf measured at the widest point in centimeters.
17. Leaf Color—Color of the leaves 1–2 weeks after pollination. (Light green, medium green, dark green, very dark green).
18. Leaf Marginal Waves—Rated for waves on the leaf margin 1–2 weeks after pollination (none, few, many).
19. Leaf Longitudinal Creases—Rated for creases on the leaf surface 1–2 weeks after pollination (absent, few or many).
20. Leaf Sheath Anthocyanin—Rated 1–2 weeks after pollination (absent, basal-weak, basal strong, weak, strong).
21. Leaf Sheath Pubescence—Rated 1–2 weeks after pollination (light, medium or heavy).
22. Tassel Length—Measured from the base of the bottom tassel branch to the tassel tip in centimeters.
23. Tassel Spike Length—Measured from the base of the top tassel branch to the tassel tip in centimeters.
24. Peduncle Length—Measured from the base of the flag leaf to the base of the bottom tassel branch in centimeters.
25. Tassel Branch Number—Number of primary tassel branches.
26. Tassel Branch Angle—Angle of an average tassel branch to the main stem of the tassel; less than 30° =upright, 30°–45° =intermediate, greater than 45° =lax.
27. Tassel Attitude—Observed after pollination as open or compact.
28. Tassel Glume Color—Color of glumes at 50% shed (green, red or purple) .
29. Tassel Glume Band—Closed anthocyanin ring at base of glume (absent or present) .
30. Tassel Anther Color—Color of anthers at 50% shed (green-yellow, yellow, pink, red, purple).
31. 50% Shed Standard GDUS—Standardized GDUS are adjusted for the location and year affects (1989 DeKalb factor=−115. 1989 Illiopolis factor=+24).
32. 50% Silk Standard GDUS—Standardized GDUS are adjusted for the location and year affects (1989 DeKalb factor=−115, 1989 Illiopolis factor=+24).
33. Uniformity—Rated throughout the growing season based on variability in plant & ear height, tassel type, anther and silk color (scale of 1–5; 1=best).
34. Husk Bract—Length of an average husk leaf (short, medium or long).
35. Husk Cover—Distance from the tip of the ear to the tip of the husks (exposed ear=0.0).
36. Husk Opening—Husks are rated for tightness at harvest stage (tight, intermediate or open).
37. Husk Color Fresh—Color of husks 1–2 weeks after pollination (green, red or purple).
38. Husk Color Dry—Color of husks at harvest stage (buff, red or purple).
39. Shank Length—Length of the ear shank measured in centimeters.
40. Shank Internode Number—Number of internodes on the ear shank.
41. Ear Height—Measured from the soil to the base of the primary ear in centimeters.
42. Ears/Stalk—The number of ears with grain on each plant.
43. Ear Silk color—color of silk observed 2–3 days after silks emerge (green-yellow, yellow, pink, red, purple).
44. Ear Position—Rated at harvest stage (upright, horizontal, pendent).
45. Ear Weight—Weight of the ear expressed in grams.
46. Ear Length—Length of the ear measured in centimeters.
47. Ear Diameter—Diameter of the ear at the midpoint measured in centimeters.
48. Ear Shape—Observed as conical, semi-conical or cylindrical.
49. Kernel Row Number—Number of rows on one ear.
50. Kernel Row Direction—Observed as straight, slightly curved, spiral or indistinct (scattered).
51. Kernel Number per Row—Number of kernels in one row.
52. Kernel Cap Color—Color of the kernel cap observed at dry stage (white, lemon-yellow, yellow, orange).
53. Kernel Side Color—Color of the kernel side observed at dry stage (white, pale yellow, yellow, orange, red, brown).
54. Kernel Endosperm Type—Observed as normal, waxy, opaque.
55. Kernel Endosperm Color—Observed as white, pale yellow, yellow.
56. Kernel Weight/1000k—Weight of 1000 kernels expressed in grams.
57. Kernel Length—Distance from the cap to the pedicel measured in millimeters.
58. Kernel Width—Distance across the flat side of the kernel measured in millimeters.
59. Kernel Thickness—Distance across the narrow side of the kernel measured in millimeters.
60. Kernel Type—Observed as dent, flint, intermediate.

61. Cob Diameter—Diameter of the cob at the midpoint measured in centimeters.
62. Cob Strength—Cobs are mechanically broken and rated as either strong or weak.
63. Cob Color—Color of the cob (white, pink, red, brown, purple).
64. Shelling Percentage—Shelled grain weight divided by the sum of grain and cob weight, expressed as a percentage.
65. Test Weight—U.S. Government standards are that 56 lbs equals 1 bushel, with a 15% moisture content.
66. GDU Shed—The number of growing degree units (GDU) required for an inbred line or hybrid to shed pollen from the time of planting.

GDU=[(Max+Min) daily temperature ° F.]−50° F./2

Where maximum daily temperature must not exceed 85° F. and minimum must not be below 50° F.

67. Selection Index—A single measure of a hybrid's worth based on information for up to five traits (always includes yield).
68. Yield—Actual bushels/acre at harvest adjusted to 15.5% moisture.
69. Moisture—Per cent moisture of the grain at harvest.
70. Stay Green—A measure of plant health at the time of maturity of the ear leaves.
71. Dropped Ears—The per cent of plants that did not drop ears prior to harvest.

REFERENCES

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

Reference 1. Armstrong and Green, (1985). "Establishment and Maintenance of Friable, Embryogenic Maize Callus and the Involvement of L-Proline," *Planta,* 164:207–214.

Reference 2. Chu, C. C., Wang, C. C., Sun, C. S., et al. (1975). *Scientia Sinica* 18:659–668.

Reference 3. Coe, E. H., et al (1988) "The Genetics of Corn and Corn Improvement", 3rd. ed., Vol. 18, Sprague and Dudley (eds) 87:258.

Reference 4. Duvick, D. N. (1984) "Genetic Contribution to Yield Gains of U.S. Hybrid Maize—19-30–1980," Genetic Contribution to Yield Gains of Five Major Crops, pp. 15–48.

Reference 5. Finkle, B. J., Ulrich, J. M., Rains, W., et al. (1985). *Plant Sci.* 42:133–140.

Reference 6. Goodman, M. and Stuber, C., "Genetic Identification of Lines and Crosses Using Isoenzyme Electrophoresis," Proceedings of the Thirty-Fifth Annual Corn and Sorghum Industry Research Conferences, Chicago, 1980.

Reference 7. Gordon-Kamm, W. et al., (1990) "Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants," *The Plant Cell,* V. 2, 603–618.

Reference 8. Hauptmann, R. M., Vasil, V., Ozias-Aikins, P., et al. (1988). *Plant Physiol.* 86:602–606.

Reference 9. Klein, T. M., Kornstein, L., Sanford, J. C., et al. (1989). *Plant Physiol.* 91:440–444.

Reference 10. Murashige, T. and Skoog, F. (1962). *Plant Physiol.* 15:473–497.

Reference 11. Rhodes, C. A., Pierce, D. A., Mettler, F. J., et al. (1988). *Science* 240:204–207.

Reference 12. Roberts, *Nuc. Acids Res.* 10:117–144 (1982).

Reference 13. Troyer, A. F. (1990) "A Retrospective View of Corn Genetic Resources" *Journal of Heredity,* 81:17–24.

Reference 14. Withers, L. A., King, P. J. (1979). *Plant Physiol.* 64:675–678.

What is claimed is:

1. A hybrid corn plant designated DK671 formed by the crossing of inbred corn plants B73HT and MBUB.
2. A seed of the corn plant designated DK671.
3. A hybrid corn plant having all the physiological and morphological characteristics of the corn plant designated DK671.
4. A tissue culture of regenerable cells from DK671.

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